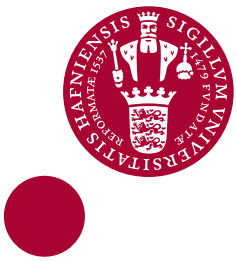

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# Secretion of Alpha-Hemolysin by *Escherichia coli* Disrupts Tight Junctions in Ulcerative Colitis Patients

**Hengameh Chloé Mirsepasi-Lauridsen, PhD[1,2,6], Zhengyu Du, MSc[3,6], Carsten Struve, PhD[1], Godefroid Charbon, PhD[2], Jurgen Karczewski, PhD[3], Karen Angeliki Krogfelt, PhD[1], Andreas Munk Petersen, PhD[1,4,5] and Jerry M Wells, PhD[3]**

**OBJECTIVES: The potential of *Escherichia coli* (*E. coli*) isolated from inflammatory bowel disease (IBD) patients to damage the integrity of the intestinal epithelium was investigated.**

**METHODS: *E. coli* strains isolated from patients with ulcerative colitis (UC) and healthy controls were tested for virulence capacity by molecular techniques and cytotoxic assays and transepithelial electric resistance (TER). *E. coli* isolate p19A was selected, and deletion mutants were created for alpha-hemolysin (α-hemolysin) (*hly*) clusters and cytotoxic necrotizing factor type 1 (*cnf1*). Probiotic *E. coli* Nissle and pathogenic *E. coli* LF82 were used as controls.**

**RESULTS: *E. coli* strains from patients with active UC completely disrupted epithelial cell tight junctions shortly after inoculation. These strains belong to phylogenetic group B2 and are all α-hemolysin positive. In contrast, probiotic *E. coli* Nissle, pathogenic *E. coli* LF82, four *E. coli* from patients with inactive UC and three *E. coli* strains from healthy controls did not disrupt tight junctions. *E. coli* p19A WT as well as *cnf1*, and single loci of *hly* mutants from cluster I and II were all able to damage Caco-2 (Heterogeneous human epithelial colorectal adenocarcinoma) cell tight junctions. However, this phenotype was lost in a mutant with knockout (Δ) of both *hly* loci ($P < 0.001$).**

**CONCLUSIONS: UC-associated *E. coli* producing α-hemolysin can cause rapid loss of tight junction integrity in differentiated Caco-2 cell monolayers. This effect was abolished in a mutant unable to express α-hemolysin. These results suggest that high Hly expression may be a mechanism by which specific strains of *E. coli* pathobionts can contribute to epithelial barrier dysfunction and pathophysiology of disease in IBD.**

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**Subject Category:** Inflammatory Bowel Disease

## INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are two different forms of chronic inflammatory bowel disease (IBD), the etiology of which is still unknown. CD and UC are distinguished by their clinical manifestations and inflammatory profiles.[1] UC is a chronic inflammatory disorder of the colorectal mucosa, whereas CD is a chronic, segmentally localized granulomatous disease of the gastro-intestinal tract CD may even affect non-intestinal tissue such as lymph nodes and skin. Clinical practice has seen that in both diseases chronicity is interrupted by acute flares, bloody diarrhea, relapses, and remission. IBD can appear at any age, however, most often in the third decade of life.[2] The highest reported prevalence values for IBD are in Europe (UC, 505 per 100,000 persons; CD, 322 per 100,000 persons).[3]

Genome-wide association studies in IBD have identified genetic polymorphisms contributing to susceptibility to IBD. Many of these gene polymorphisms are associated with pathways involved in intestinal homeostasis, linking host genetics to deregulated host responses to the microbiota.[4] The concordance rate among monozygotic twins was 6.3% for UC and 58.3% for CD.[5] This clearly indicates a role of genetic factors in CD, but also indicates an important role for environmental factors, particularly in UC. An abnormal microbiota composition and decreased complexity of the gut microbial ecosystem (commonly referred to as dysbiosis) are common features in patients with CD or UC.[6] These observations have fueled efforts to identify opportunistic gut pathogens (or pathobionts) that may have a role in the pathogenesis of IBD.

*Escherichia coli* pathobionts exhibiting pathogen-like behaviors are more frequently cultured from IBD patients with active disease due to their selective growth advantage in inflammatory conditions.[7] Moreover, adherence of the B2 phylotype *E. coli* to human intestinal epithelial cells is mediated through the type 1 pili interaction with mannosylated carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6). Interestingly, CEACAM6 expression by cultured intestinal cells was shown previously to be upregulated after treatment with interferon γ and tumor necrosis factor-α.[8] These findings indicate that inflammatory conditions in the gut support *E. coli* colonization via increased CEACAM6 expression and offer an explanation for their more frequent isolation from patients with active disease.

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Among the Proteobacteria, adherent invasive variants of the B2 phylogroup *E. coli* (AIEC) have been proposed to have a role in the pathophysiology of IBD,[9] owing to their capacity to adhere to intestinal epithelial cells, to invade intestinal epithelial cells via a macropinocytosis-like process, and to survive and replicate intracellularly in epithelial cells and macrophages.[10] Others have, likewise, found increased numbers of B2 phylogroup *E. coli* isolated from IBD patients.[11] Petersen *et al.*[12] showed that *E. coli* isolates from fecal samples of primarily UC patients with active disease frequently belong to the B2 phylogenetic group and harbor genes commonly associated with extra-intestinal pathogenic *E. coli* (ExPEC) causing urinary tract infection and meningitis.[13]

Recently, a hemolysin (Hly) producing *E. coli* strain was shown to induce localized defects in epithelial integrity colonic cell monolayers and rat colon tissue *ex vivo.* Additionally, wild-type (WT) and colitis susceptible IL-10$^{-/-}$ mice colonized with an HlyA-expressing *E. coli* had elevated inflammation scores and an increased epithelial permeability compared with mice colonized with the HlyA-deficient mutant. Furthermore, qPCR analysis revealed that lesions (focal leaks) in mucosal samples from the human colon were associated with 10-fold higher levels of *hlyA* DNA, suggesting that Hly-expressing *E. coli* have a role in the pathology of intestinal inflammation in IBD.[14]

The aim of this study was to extend the above observations to isolates of B2 phylogroup *E. coli* from IBD patients by testing their effects on permeability, tight junction stability, and viability of human intestinal cell epithelial monolayers cultured *in vitro*. For comparison, we also tested the effect of prototype AIEC strain LF82 and the probiotic *E. coli* Nissle on permeability and viability of polarized human Caco-2 cells. As some strains of B2 phylogroup *E. coli* isolated from UC patients also possess a gene encoding cytotoxic necrotizing factor type 1 (*cnf1*), we investigated the role of *cnf1 and hlyA* in causing epithelial damage by the construction and testing of genetic mutants in cellular assays.

## METHODS

**Study material and ethics.** Permission for the study was obtained from the Regional Ethics Committee for Copenhagen County Hospitals (Permission no. KA03019), and all participants gave their informed written consents. Healthy controls were recruited among medical students. All controls had a completely normal distal colon as visualized by video sigmoidoscopy (left side colon) at study entry. None of the controls had experienced diarrhea, blood in stools, or abdominal pain or any other abdominal discomfort when the stool sample was submitted. Patients with IBD were diagnosed according to standardized criteria,[15,16] which included confirmation of inflammation by video sigmoidoscopy and a fresh set of negative stool cultures for common pathogens including *Clostridium difficile*.

Detailed information regarding extent of disease and current medication among the included patients has previously been described,[12] neither controls nor patients had received antibiotics within the last 2 months before inclusion and all patients had an established diagnosis of IBD before inclusion in our study.

Fecal samples were cultured at Statens Serum Institut (SSI): bacteriological analysis, *E. coli* phenotypic characterization, determination of phylogenetic group, and ExPEC virulence gene detection were performed as described previously in Petersen *et al.*[12] *E. coli* clinical isolates p7, p10, p13, p19A, p22, p25, p26, p27, and p32; healthy control *E. coli* isolates C2, C4, and C6 were characterized by PCR for virulence genes (data not shown) in this study. The probiotic *E. coli* Nissle 1917 and the pathogenic *E. coli* LF82 (ref. 17) were used as a negative and positive control, respectively.

Patient characteristics and diseases association and location are described in Table 1.

**Cell infection assay and measurement of transepithelial electric resistance.** Heterogeneous human epithelial colorectal adenocarcinoma cells (Caco-2 BBE cell line, ATCC CRL 2102) were maintained at 37 °C in a humidified 5% $CO_2$ atmosphere in Dulbecco's modified Eagle medium (DMEM;

**Table 1** Characteristics of patients and controls included in the study

| ID number | Disease association | Medication | Disease localization[a] | Gender | Age |
|---|---|---|---|---|---|
| p7 | Active UC | 5-ASA | Proctosigmoid colon | Male | 71 |
| p13 | Active UC | 5-ASA Azathioprine, Prednisolone, | Pancolitis | Male | 39 |
| p19A | Active UC | 5-ASA, Azathioprine | Proctosigmoid colon | Male | 71 |
| p22 | Active UC | 5-ASA | Proctosigmoid colon | Female | 40 |
| p25 | Active UC | 5-ASA | Pancolitis | Male | 34 |
| p10 | Inactive UC | 5-ASA | Proctosigmoid colon | Female | 40 |
| p26 | Inactive UC | 5-ASA | Proctosigmoid colon | Male | 53 |
| p27 | Inactive UC | Azathioprine | Rectum | Male | 37 |
| p32 | Inactive UC | None | Pancolitis | Female | 40 |
| C2 | Healthy | None | None | Female | 25 |
| C4 | Healthy | None | None | Male | 24 |
| C6 | Healthy | None | None | Female | 29 |
| LF82 | Crohn's disease | None | Ileum | Not known | Not known |
| *E. coli* Nissle | Healthy | None | None | Male | Not known |

UC, ulcerative colitis; 5-ASA, 5-aminosalicylic acid.
[a]Present when active disease, previous when inactive disease.

Invitrogen, Paisley, UK) containing Glutamax, 10% heat-inactivated fetal bovine serum (PAA Laboratories, Colbe, DE), 100 U/ml penicillin, 100 µg/ml streptomycin (PenStrep, Sigma, St. Louis, MO), 1% non-essential amino acids (Lonza, Basel, Switzerland), and 1% L-glutamine.

Caco-2 cells (between passages 55 and 60) were seeded at a density of $2.6 \times 10^5$ cells/cm$^2$ in a 24-transwell system containing Tissue Culture-treated filter (0.4 µm pore size, BD Biosciences Falcon type # 353494, Erembodegem, Belgium) and grown for 16 days until they differentiated into polarized monolayers. After 14 days, the transepithelial electric resistance (TER) reached 600–800 Ohms/cm$^2$ (Volt/Ohm meter; World Precision Instruments, Sarasota, FL).

For bacterial co-incubation experiments, the medium was removed by aspiration from the Transwell filters, and the filters were inserted in the cellZscope apparatus (Nanoanalytics, Münster, DE). Cell-culture medium without antibiotics was then added to the upper chambers (450 µl) and lower chambers (800 µl), and the apparatus was placed in a humidified incubator at 37°C containing 5% $CO_2$/95% $O_2$ atmosphere for 2 h before the addition of bacteria. Bacteria were grown overnight in LB (Luria broth) media at 37 °C, centrifuged, resuspended in DMEM, and added to the upper chambers (filter) in the cellZscope at a multiplicity of infection (MOI) of 10. TER measurements were recorded continuously for up to 24 h, and TER values were normalized to the initial TER value (100%) and absolute TER is mean of four independent measurements. As a control, TER was measured for uninfected Caco-2 cell monolayers (controls in figures). Three independent experiments were performed for p19A WT and its mutant strains.

**Detection of occludin by immunofluorescence and western blotting.** To visualize the effect of bacteria on Caco-2 cells, occludin was detected by immunofluorescence microscopy[18] and western blotting. Caco-2 cell monolayers were grown as described above and infected with bacteria for up to 15 h at a MOI of max. 100. Caco-2 cell monolayers were either fixed for immunofluorescence or lysed in 100 µl of lysis buffer (Promega, Madison, WI) on ice for 5–10 min. The cell lysate was centrifuged at 13,000 *g* for 12 min to pellet debris, and the supernatant was used for western blotting. Fifty micrograms of Caco-2 cell proteins were resolved by 10% SDS-PAGE and transferred onto 0.2 µm polyvinylidene fluoride (PVDF) membranes. Membranes were blocked for 1 h with 3% non-fat milk powder diluted in 0.05% Tween-20 (TBST), then incubated with primary antibody in 3% non-fat milk powder diluted in TBST overnight at 4 °C. Hereafter, membranes were visualized with secondary (Sigma-Aldrich, GmbH, Germany, DE) antibody for 1 h at room temperature. Rabbit polyclonal anti-actin antibody (A2066; Sigma-Aldrich) and rabbit anti-Occludin antibody (ABT146 Merck KGaA, Darmstadt, Germany, DE) were used in this study.

**Hemolysin assay.** The presence of α-hemolysin was demonstrated on 5% sheep blood agar plates (SSI no. 31349, Statens Serum Institut, Diagnostica, Hillerød, Denmark) after 3–4 h of incubation at 37 °C as opposed to enterohemolysin, which was detectable only after overnight incubation at 37 °C.

**Hemolysis determination by titration assay.** Defibrinated horse blood (SSI no. 23699 Statens Serum Institut, Diagnostica) was washed twice in hemolysis buffer (0.0077 M Tris-HCl, 0.137 M NaCl and 0.02 M $CaCl_2$ pH 7.4) and centrifuged at 300 *g* for 5 min. Washed red blood cells were resuspended in hemolysis buffer to a final concentration of 2% red blood cells. Overnight bacterial culture (approx. CFU (colony forming units) $2 \times 10^8$), 5 ml LB, 37 °C, was centrifuged; and both the bacterial pellet and the bacterial growth supernatant were tested for hemolytic activity. The bacterial pellet was resuspended in 5 ml hemolysis buffer. Two-fold serial dilutions (1:2 to 1:1024) in microtiter plates of either 150 µl of bacterial suspension or 150 µl of bacterial supernatant were performed in phosphate-buffered saline (pH 7.4; Sigma-Aldrich, St. Louis, MO), and finally 150 µl 2% red blood cells suspension was added and incubated for 2 h at 37 °C. After incubation, the plate was centrifuged for 10 min at 700 *g*, 150 µl of supernatant was transferred to a new microtiter plate, and the OD (optical density) measured at 562 nm. Hemolytic titration assays were performed at least twice with essentially the same results. Hemolysis buffer and phosphate-buffered saline were used as negative controls.

**Construction of genetic deletion mutants.** Isogenic mutants of the *E. coli* clinical isolate p19A were constructed by allelic exchange with antibiotic resistance encoding cassettes using the λ-Red recombinase method as previously described.[19] All primers used are shown in Table 2. For deletion of the *hly* cluster, 289- bp and 422- bp regions flanking the *hly* gene cluster were amplified by PCR using the primer pairs UphlyC-F/UphlyC-R and DwhlyD/DwhlyD-R and added to a kanamycin cassette. To construct the double *hly* mutant (Δ*hlyI*, Δ*hlyII*), the λ-Red procedure was repeated on the single *hly* mutant (Δ*hlyI*) using a tetracycline resistance encoding cassette PCR amplified by primers 379 and 380 containing 50-bp overhangs homologous to up- and downstream regions, respectively, of the *hly* gene cluster. The *cnf1* cluster was deleted using a tetracycline resistance encoding cassette PCR amplified by the use of primers Upcnf-F and Dwcnf-R containing 50-bp overhangs homologous to up- and

**Table 2** Primers used for construction of mutants

| Primer name | Sequence 5′ to 3′ |
|---|---|
| UphlyC-F | CGGGCTAACCAATATGCT |
| UphlyC-R | GAAGCAGCTCCAGCCTACACCCTCCGTGAAATTCTGATACT |
| DwhlyD-F | GGACCATGGCTAATTCCCATAAGAAAAGAGCAGAGCGA |
| DwhlyD-R | GTAACAACCCCACCTTCA |
| 379 | CACCACGAGTTAATAACTGAAGTAAAAAACAAGACAGATTT-CAATTTTTCATTAACAGGCAAGAATTGCCGGCGGAT |
| 380 | CTGTTAGTCTGACTGTAACTGATATAAGTAACTGTATAAACTT-TCTGGTTCGGTATTTCACACCGCATAGC |
| Upcnf-F | GATTAGGTATTCTGATAAGGTGTAGTAAAATATTAATCTT-CACA-GAGGAGCAAGAATTGCCGGCGGAT |
| Upcnf-R | GCGCTAACAAAACAGCACAAGGGTAACTTATAACAATGGC-CAAT-AAATAATTTCCCGGGTATTTCACACCGCATAGCAG |
| hlyA-F | ACCTTGTCAGGACGGCAGAT |
| hlyA-R | CCGTGCCATTCTTTTCATCA |
| RrpoA1 | TTGATATCGAGCAAGTGAGTTCG |
| RrpoA2 | GCATCGATGAGAGCAGAATACG |

downstream regions the *cnf1* gene, respectively. Allelic replacement was mediated via the thermo-sensitive helper plasmid pKOBEGApra, encoding λ-Red recombinase functions. Allelic replacements were verified by PCR.

**Quantification of hemolysin expression.** Total RNA was phenol/chloroform extracted from LB growing cultures at $OD_{600}$ 0.8 (approx. $5 \times 10^6$ CFU/ml) followed by DNase I digestion (# EN0525; Thermoscientific, Herlev, Denmark). The RNA was then purified using Qiagen column (cat. no. 74104) treated with a dsDNase (# EN0771; Thermoscientific) and directly used for cDNA preparation using a First Strand cDNA synthesis kit (# K0702; Thermoscientific). For the amplification of *hlyA*, primers *hlyA* forward and *hlyA* reverse were used (Table 2).[20] The gene *RpoA* was used as a housekeeping/reference gene and amplified by primer pair RrpoA1 RrpoA2 (Table 2).[21] The quantitative-PCR assay was performed using Takara SYBR Premix Ex Taq II (RR820A, Mountain View, CA) in a BioRAD CFX96 (Hercules, CA). The PCR was performed using the manufacturer's recommendations: preheating at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s for elongation.

**Cytotoxicity by neutral red assay.** Caco-2 BBE cell line (between passages 60 and 68) was maintained at 37 °C in a humidified 5% $CO_2$/95% $O_2$ atmosphere, in DMEM containing Glutamax, 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids, and 1% L-glutamine for 7 days. After 7 days, the media were removed from the confluent cell layer by aspiration, and the monolayer was washed twice with phosphate-buffered saline. Trypsinated cell suspension was seeded in 24-transwell plates (seeding density of $0.05 \times 10^6/cm^2$) and incubated overnight before co-incubation with bacteria. Caco-2 cells were infected with an overnight culture of *E. coli* grown in DMEM at an MOI of 10 and maintained at 37 °C in a humidified 5% $CO_2$/95% $O_2$ atmosphere for 4 h. The monolayer was then washed once with DMEM, and then DMEM containing 50 μg/ml neutral red (N4638, Sigma-Aldrich, Brøndby, Denmark) was added and incubated at 37 °C for 30 min. Hereafter, the cells were washed rapidly with a suspension containing 40% formaldehyde and 10% $CaCl_2$. Neutral red was extracted with 1% acetic acid-50% ethanol and quantified in a spectrometer (OD 450 nm). The amount of extracted neutral red is expressed as a percentage of the amount recovered from uninfected cells.

**Statistics.** The software "GraphPad Prism 5" was used for statistical analysis. TER and hemolytic titration results were analyzed using the two-way ANOVA test when compared with blank or negative control. Neutral Red test results were analyzed using the one-way ANOVA test.

## RESULTS

**Hemolytic strains of *E. coli* isolated from IBD patients with active disease disrupt the epithelial cell barrier integrity tested by TER.** In this study, we investigated the effect of twelve *E. coli* strains isolated from nine patients with IBD and three control subjects on intestinal epithelial integrity, using TER measurements of Caco-2 cell monolayers grown in Transwells (Table 3). Three of the five phylotype B2 *E. coli* strains, p7, p19A, and p22, isolated from UC patients with active disease induced a rapid decrease in TER at an MOI of 10, starting after about 2 h and resulting in a complete loss of TER by 6 h (Figures 1a and b). All four *E. coli* strains isolated from patients with inactive UC or healthy controls decreased the TER after 10–12 h, which was similar for the probiotic *E. coli* Nissle and the adhesive and invasive *E. coli* (AIEC) strain LF82 (isolated from an ileal biopsy of a patient with CD[11]) (Figures 1a–c). The loss of TER after about 10–15 h is due to the growth of *E. coli* and acidification of the medium. As expected, the TER of untreated Caco-2 cell monolayers was not significantly changed over the 20 h of incubation. Isolates p7, p19A, and p22 were identified as the only α-hemolytic strains among those tested, therefore implicating α-hemolysin in the disruption of TER (Table 3).

**IBD-associated strain p19A contains *cnf1* and two *hly* gene clusters.** Among the α-hemolysin-positive strains from patients with active UC, p19A was chosen for further investigation. In a previous study, we have shown that *E. coli*

**Table 3** Origin, molecular, and physiological characteristics of *E. coli* strains used in this study

| *E. coli* strain | Phylogenetic group | Disease association | Hemolytic activity | TER reduction (h) | Two-way ANOVA (TER: 5-12 h) |
|---|---|---|---|---|---|
| p7 | B2 | Active UC | Alfa (<4 h) | 6 | $P<0.05$*** |
| p13 | B2 | Active UC | None | >10 | ns |
| p19A | B2 | Active UC | Alfa (<4 h) | 6 | $P<0.05$*** |
| p22 | B2 | Active UC | Alfa (<4 h) | 6 | $P<0.05$*** |
| p25 | B2 | Active UC | Ent (24 h) | >10 | ns |
| p10 | A | Inactive UC | None | >10 | ns |
| p26 | A | Inactive UC | Ent (24 h) | >10 | ns |
| p27 | A | Inactive UC | Ent (24 h) | >10 | ns |
| p32 | B2 | Inactive UC | None | >10 | ns |
| C2 | A | Healthy | None | >10 | ns |
| C4 | B1 | Healthy | None | >10 | ns |
| C6 | D | Healthy | None | >10 | ns |
| LF82 | B2 | Crohn's disease | None | >10 | ns |
| *E. coli* Nissle | B2 | Healthy | None | >10 | ns |

ANOVA, analysis of variance; TER, transepithelial electric resistance; UC, ulcerative colitis.
***Statistical significant.

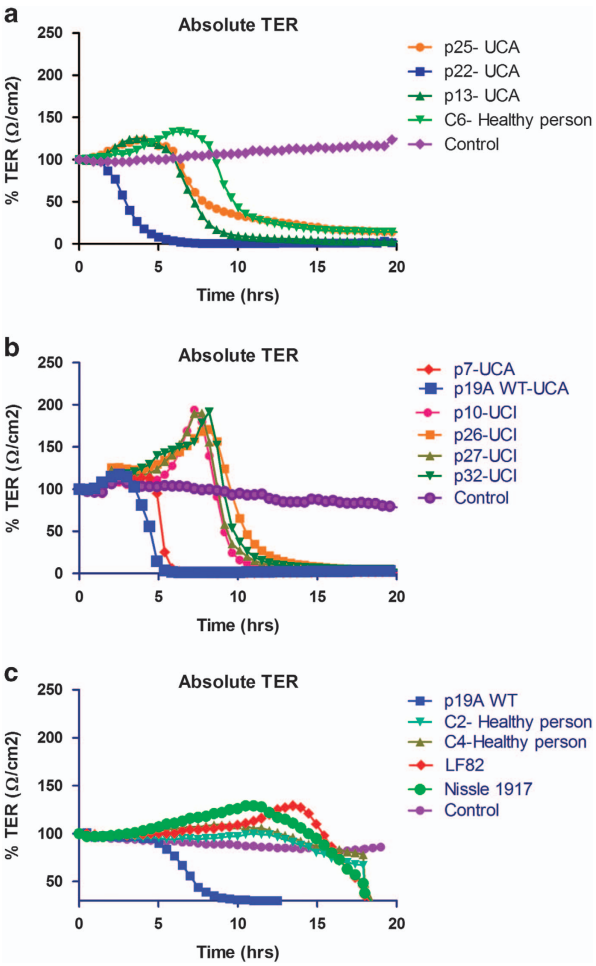


**Figure 1** Effect of *E. coli* clinical isolates from inflammatory bowel disease (IBD) patients and controls on a monolayer of Caco-2 cells measured by transepithelial electric resistance (TER). (**a**) *E. coli* strains p13, p22, and p25 from patients with active ulcerative colitis (UCA) revealed that p22 disrupted the epithelial cell barrier in less than 6 h after co-incubation with Caco-2 cells, while strains p13 and p25, and *E. coli* C6 from a healthy control did not disrupt the epithelial barrier until after 10 h of co-incubation. (**b**) *E. coli* strains p7 and p19A wild type (WT) from patients with UCA disrupted the epithelial cell barrier in less than 6 h after co-incubation with Caco-2 cells. *E. coli* p10, p26, p27, and p32 from patients with inactive ulcerative colitis (UCI) did not disrupt the epithelial barrier until after 10 h of co-incubation. (**c**) *E. coli* strain p19A WT from UCA patient was compared with the adherent invasive Crohn's disease (CD) associated *E. coli* LF82, the probiotic *E. coli* Nissle and two *E. coli* strains, C2 and C4, isolated from healthy controls, it is seen that the probiotic and control isolates did not disrupt epithelial barrier until after 15 h of co-incubation. Simultaneously with all experiments presented (**a–c**) control TER of the media was performed on Caco-2 cells without addition of bacteria. TER values were normalized to the initial TER value (100%). Absolute TER values are mean of four measurements.

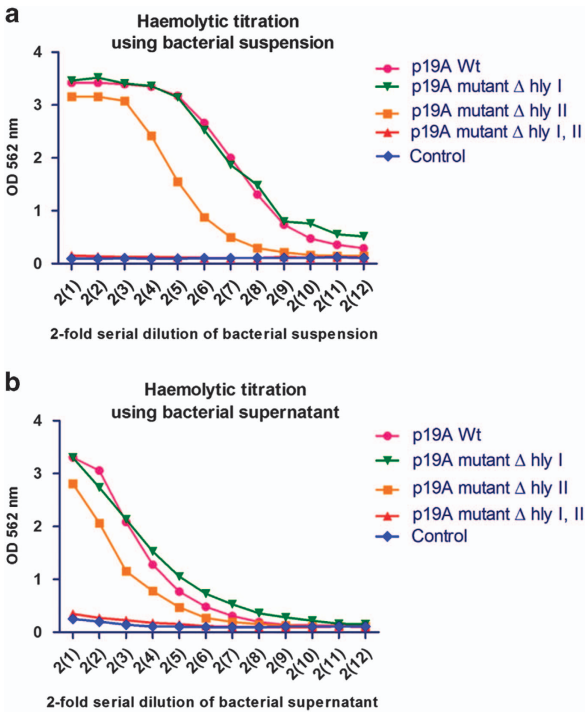


**Figure 2** Hemolysin activity of clinical isolate p19A wild type (WT) from active ulcerative colitis (UCA) patient and its isogenic mutants. (**a**) Bacterial cell suspension of clinical isolate p19A WT and the two single hemolysin mutants (p19A Δ*hlyI* and p19A Δ*hly*II) showed almost strong hemolytic activity, while in p19A hemolysin double mutant (p19A Δ*hly* I and II) the activity was completely hampered. We used buffer as a control. (**b**) Bacterial growth supernatant of the above cultures revealed the same hemolytic activity while the double mutant had abolished activity. We used growth medium as a control. OD, optical density.

strain p19A belongs to the phylogenetic group B2, and harbors *cnf1* and *hly* genes.[22] To determine the possible role of *E. coli hly* and *cnf1* in barrier disruption, deletion mutants of the individual toxin-encoding genes were constructed. The first *hly* mutant constructed (Δ*hlyI*) was still hemolytic, indicating that p19A contained two *hly* clusters. The presence of two *hly* clusters has been reported for some ExPEC isolates, and in these strains one of the *hly* clusters is often located upstream of the *cnf1* gene.[23] Indeed PCR analysis of strain p19A revealed that the intact *hly* cluster remaining in the Δ*hlyI* mutant was located upstream of the *cnf1* gene. Thus, mutants of p19A lacking the second *hly* cluster (Δ*hlyII*) and also both *hly* clusters were constructed (Δ*hlyI*, *II*).

Hemolytic titration assays were performed with bacteria and bacterial culture supernatants in order to investigate the hemolytic activity of clinical isolate *E. coli* p19A WT and the *hly* and *cnf1* mutants. The hemolytic activity was only completely abolished in the double mutant lacking *hly* clusters I and II ($P<0.05$) (Figure 2). Deletion of *hly* cluster II only partially decreased hemolytic activity, compared with the WT, suggesting that *hly* cluster I does contribute to the overall hemolytic activity of the WT strain, despite the fact that no reduction in hemolysis was observed in the *hly* cluster I mutant.

RT-PCR was used to quantify the relative amounts of the *hly* transcript in *E. coli* p19A WT and the different *hly* deletion mutants, using *rpoA* transcripts as an internal control. The relative expression of *hly* was twofold higher in the WT p19A than in the Δ*hlyI* mutant and fourfold higher than in the Δ*hlyII* mutant. As expected, only the double Δ*hlyI*, *II* mutant of p19A lacked *hly* gene expression (Figure 3).

***hly* expression in IBD-associated strain p19A causes rapid loss of epithelial integrity.** To study the effect of *hly* and *cnf1* expression on the intestinal epithelial barrier integrity, TER measurements were performed with p19A

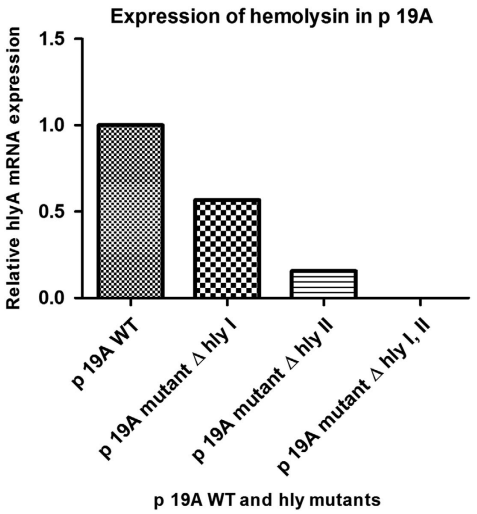


**Figure 3** Quantification of hemolysin expression in clinical isolate p19A. It is clearly seen that p19A wild type (WT) expresses more *hly* compared with the single mutants. Expression of hemolysin is completely abolished in the double mutant p19A Δ*hly I* and *II*. Relative *hlyA* mRNA was measured by quantitative RT-PCR. The *rpoA* mRNA level was used as an internal quantitative control.

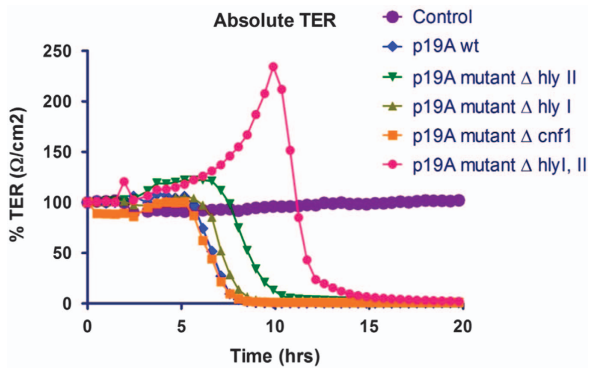


**Figure 4** Effect of clinical isolate *E.coli* p19A and its hemolysin deletion mutants on a monolayer of Caco-2 cells measured by transepithelial electric resistance (TER). Wild type (WT) p19A and its single hemolysin- and *cnf1*-deletion mutants all disrupted the epithelial barrier in less than 6 h, whereas the p19A Δ*hlyI, II* double deletion mutant did not have any effect on TER. As a control, no bacteria were added to the Caco-2 cells.

WT and its deletion mutants. At an MOI of 10, the WT, single mutants Δ*hlyI*, Δ*hlyII*, and Δ*cnf1* strains caused loss of TER in Caco-2 cell monolayers in less than 6 h (Figure 4). Deletion of both *hly* clusters (Δ*hlyI*, *II*) in p19A WT abrogated the rapid loss of epithelial integrity, and the effects on TER were comparable to the probiotic Nissle and other *E. coli* strains not expressing *hly* (12 h; $P<0.0001$***).

***hly* expression is linked to rapid dissolution of occludin from the tight junctions of epithelial cell monolayers.** To investigate the effect of hemolytic strains on tight junctions, Caco-2 cell monolayers were incubated with *E. coli* p19A for <1, 2, or 3 h, and then fixed and stained for occludin and nuclear DNA (Figure 5). No dissolution of occludin from the tight junction was observed with *E. coli* Nissle or the Hly-negative AIEC strain LF82. A significant reduction in the immunofluorescent staining of occludin was evident, from 2 to 3 h of co-culture with WT p19A ($P<0.001$) as confirmed by western blotting with antibodies to occludin (Figure 5). Similar results were obtained with all three Hly-producing *E. coli* strains p7, p19A, or p22, but not with the p19A Δ*hlyI*, Δ*hlyI II* double mutant demonstrating a link between Hly expression and loss of tight junction occludin (data not shown).

**Effect of p19A WT on epithelial tight junctions disruption and loss of TER is not due to cytotoxicity.** To investigate a possible cytotoxic effect of *hly* and *cnf1* genes on epithelial cells in the above assays, Neutral Red uptake by viable Caco-2 cells was measured after 4 h of co-culture with p19A WT and its mutants at the same MOI. Neutral Red assays were performed six times on 3 different days. No significant differences were found between p19A WT and mutants, which rules out the possibility that cytotoxicity of Hly or Cnf1 causes the rapid loss of occludin and a decrease in TER (Figure 6).

## DISCUSSION

The epithelial cell layer is an essential constituent of the gut and a highly specialized interface between the host and its environment. Desmosomes, adherence junctions, and tight junctions hold the cells of the intestinal epithelial layer together. Tight junctions are important in controlling paracellular permeability to ions and small molecules and preventing translocation of luminal antigens and bacteria into the lamina propria.[24] In this paper, we demonstrated that IBD-associated *E. coli* strains from UC patients who produce α-hemolysin cause disruption of epithelial tight junctions of intestinal cell monolayers, leading to the loss of TER. Three of five UC-associated *E. coli* strains (p7, p19A, and p22) isolated from patients with active UC induced a rapid loss of TER at low MOI without any loss of cell viability.

The IBD-associated strains causing loss of epithelial integrity were all of the phylotype B2 and consistent with previous reports showing an increased abundance of the phylotype B2 *E. coli* in UC and CD patients with active disease.[11,12,25,26] The role of *E. coli* pathobionts in the pathophysiology in IBD was attributed to their capability to adhere and invade epithelial cells and replicate in macrophages, and the most well-studied prototype strain is LF82. In contrast to p19A, strain LF82 does not cause rapid dissolution of epithelial tight junctions, clearly indicating that the phylotype B2 of UC-associated strains differs markedly in pathogenic mechanisms. The type 1 fimbriae of AIEC were shown to bind to CEACAM6, which is expressed at higher levels in inflamed intestinal epithelial cells of IBD patients.[27] Our UC-associated *E. coli* p19A strain has the same capacity as LF82 to adhere to epithelial cells (data not shown).

All the UC-associated *E. coli* strains that caused loss of tight junctions in epithelial cell monolayers were hemolytic. Four types of hemolysin have been demonstrated in *E. coli*: alpha-hemolysin (HlyA), plasmid- and phage-carried enterohemolysin (EhxA and HlyA) and silent hemolysin (SheA); EhxA and HlyA belong to the RTX (repeats in toxin) related family, which

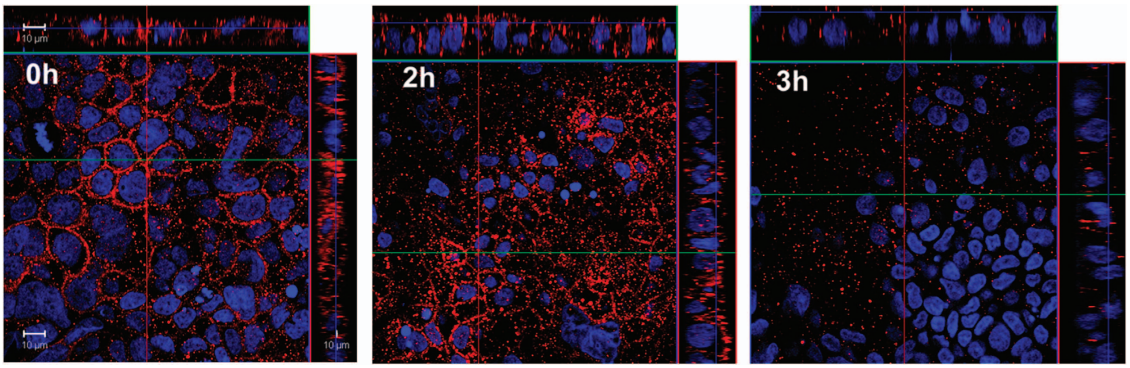


**Figure 5** Disruption of occluding on Caco-2 cells after incubation with clinical isolate p19A. Confocal images of Caco-2 cell monolayers stained for occludin (red) and nuclei (blue) after apical incubation with *E. coli* p19A (MOI: 50) after 2 and 3 h ($P < 0.01$ and $P < 0.001$, respectively). MOI, multiplicity of infection.

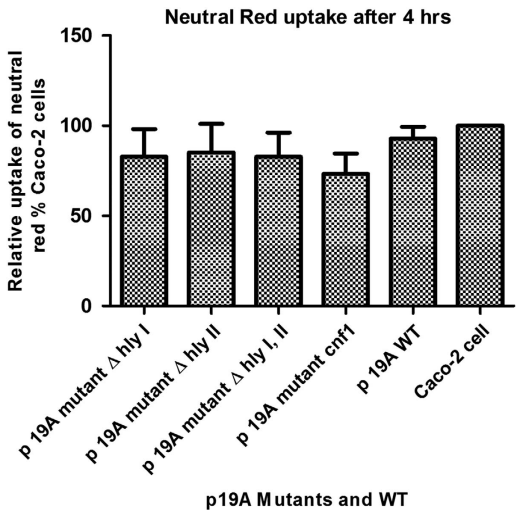


**Figure 6** Cytotoxicity of clinical isolate p19A and its hemolysin mutants on Caco-2 cells. No significant differences in Caco-2 cell viability were found between the p19A wild type (WT) and mutants. Neutral Red uptake in Caco-2 cells was measured after co-incubation with *E. coli* p19A WT and mutants.

lyse erythrocytes from different mammalian species.[28–30] It is known that a number of *E. coli* pathotypes, i.e., urinary tract pathogenic *E. coli*, enteropathogenic *E. coli*, and enterotoxigenic *E. coli* are all able to produce α-hemolysin.[20] The *E. coli* α-hemolysin is known to be able to lyse erythrocytes through binding to the surface protein glycoporin,[31–33] but also other cell types including leukocytic cells, bladder, and renal tubular cells in a dose-dependent manner.[25–29] Lysis of immune cells is greatly influenced by the presence of cell receptors CD11a and CD18, which are expressed on B and T cells, as well as neutrophils monocytes and dendritic cells.[30,31]

The role of HlyA in tight junction disruption was further investigated in *E. coli* strain p19A, which possessed two *hlyA* clusters as previously reported for some isolates of uropathogenic *E. coli* belonging to phylotype B2.[34] We showed that both *hlyA* gene clusters in p19A contributed to the damaging effects on the epithelial integrity, suggesting that intestinal *E. coli* strains possessing more than one *hlyA* locus may have increased pathological consequences in intestinal inflammation. Although our UC-associated strains did not induce epithelial cell apoptosis, an hly-expressing uropathogenic *E. coli* was previously shown to cause localized regions of apoptosis in HT29/B6 cell monolayers. The difference between these findings and our results may be due to the use of a higher MOI than in our study, the use of different strains, or the amount of *hlyA* expressed.[35]

Our study showed that around 50% of phylotype B2 *E. coli* isolated from UC patients can adhere to epithelial cells and disrupt epithelial tight junctions via an HlyA-dependent mechanism, provides strong evidence that this is an important novel pathogenic mechanism in UC; and distinct of AIEC LF82 in CD. Lesions in tight junctions of intestinal epithelium from IBD patients with active disease have been associated with a reduction in several tight junction proteins including claudin 1 and 4, occludin and tricellulin,[36] and the synthetic octapeptide (AT1001), which prevents the opening of tight junctions, improves colitis in susceptible IL-$10^{-/-}$ mice.[37] Further evidence for the importance of HlyA in the epidemiology of IBD comes from a previous study showing that an HlyA-producing strain of *E. coli* but not an HlyA-deficient mutant was a potentiator of inflammatory activity in the colon of susceptible IL-$10^{-/-}$ mice and monocolonized germ-free mice due to its effects on the epithelial barrier function.[14] During active UC and high inflammation and increased CEACAM6 expression, binding of specific *E. coli* is facilitated.

This study is a mandate for further investigation of epithelial barrier disruption in other UC cohorts and geographic locations. Preliminary evidence from genomic sequencing suggests that some *E. coli* strains carry large conjugative plasmids, suggesting that lateral gene transfer of *hly* loci could contribute to the spread of pathogenic traits.

A recent meta-study including 10 randomized trials from CD patients and 9 randomized trials from UC patients yielded an odds ratio of 2.17 (95% confidence interval, 1.54–3.05) in favor of antibiotic therapy.[38] These results suggest that antibiotics improve clinical outcomes in patients with IBD. Another meta-study published in 2011 by Khan *et al.*[39] concluded that antibiotic therapy may induce remission in active CD and UC, although the diverse number of

antibiotics tested means the data are difficult to interpret. This systematic review proposed further trials of antibiotic therapy in IBD.

Approaches for combating bacteria that adversely affect the barrier function (e.g., HlyA-expressing *E. coli*) might provide new treatment options for IBD. This might include antibiotic therapy, vaccination or competition by probiotic bacteria lacking HlyA and other virulence factors that can cause harm to the host.

### CONFLICT OF INTEREST

**Guarantor of the article**: Karen Angeliki Krogfelt, PhD.
**Specific author contributions**: Participated in the design of the study: Karen Angeliki Krogfelt, Andreas Munk Petersen, Hengameh Chloé Mirsepasi-Lauridsen, Zhengyu Du, Carsten Struve, Jurgen Karczewski, and Jerry M. Wells; drafted the manuscript: Hengameh Chloé Mirsepasi-Lauridsen, Andreas Munk Petersen, Jerry M. Wells, Carsten Struve, and Karen Angeliki Krogfelt; responsible for the experimental setting: Hengameh Chloé Mirsepasi-Lauridsen, Zhengyu Du, Carsten Struve, Godefroid Charbon, and Jurgen Karczewski. All authors have read and approved the final manuscript.
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## Study Highlights

### WHAT IS CURRENT KNOWLEDGE

✓ Hemolysin is known to lyse red blood cells (hence the name). A number of bacteria produce hemolysins that are cytotoxic to monocytes, lymphocytes, and macrophages.

### WHAT IS NEW HERE

✓ *E. coli* alpha-hemolysin expression disrupts tight junctions in epithelial cells.

✓ *E. coli* isolated from patients with active ulcerative colitis have two copies of the hemolysin gene.

### IMPACT ON CLINICAL PRACTICE

✓ The detection of hemolysin producing *E. coli* in UC patients with active disease suggests for a change in treatment policies, e.g., to include short-term target antibiotics against these specific *E .coli*.

✓ In favor of antibiotic therapy are recent meta-studies including randomized trials from CD and UC where antibiotic therapy induced remission in active disease.

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